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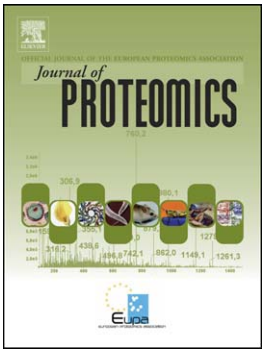

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In vivo intravascular biotinylation of *Schistosoma bovis* adult worms and proteomic analysis of tegumental surface proteins.

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Abstract

Schistosoma bovis is a blood-dwelling fluke of ruminants that lives for years inside the vasculature of their hosts. The parasite tegument covers the surface of the worms and plays a key role in the host-parasite relationship. The parasite molecules expressed at the tegument surface are potential targets for immune or drug intervention. The purpose of this work was the identification of the proteins expressed *in vivo* on the surface of the tegument of *S. bovis* adult worms. To accomplish this we used a method based on *in vivo* vascular perfusion of mice infected with *S. bovis* which allowed the labelling of the surface of the worms inside the blood vasculature. The biotinylation of parasite inside blood vessels prevents the handling of worms *in vitro* and hence possible damage to the tegument that could produce results that would be difficult to interpret. Trypsin digestion of biotinylated proteins and subsequent liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS) resulted in the identification on the *S. bovis* tegument of 80 parasite proteins and 28 host proteins. The proteins identified were compared with the findings from other proteomic studies of the schistosome surface. The experimental approach used in this work is a reliable method for selective investigation of the surface of the worms and provides valuable information about the exposed protein repertoire of the tegument of *S. bovis* in the environmental conditions that the parasite faces inside the blood vessels.

Keywords: Schistosoma bovis; tegument; proteome; vascular perfusion; biotinylation; proteome

1. Introduction

Schistosomiasis is a severe parasitic disease caused by flukes of the genus Schistosoma that affects humans and animals in many tropical and subtropical areas of the world. *Schistosoma bovis* is a blood-dwelling fluke of ruminants that, like human schistosomes, penetrates the host body through the skin until reaching a skin capillary vessel. It then migrates through the blood stream towards the lungs, where it remains for several days as a schistosomulum larva, after which it travels to the portal vein and mesenteric vessels, where it finally develops into the adult stage.

Schistosomes live for years inside the vasculature of their hosts, in contact with the vascular endothelium and the blood components involved in immune and haemostatic responses. Such a long survival time is achieved because schistosomes have developed diverse mechanisms to evade immune and haemostatic host responses, many of which are dependent on the properties of the parasite tegument [1-7].

The tegument of adult schistosomes covers the entire surface of the worms and is a unique outer-surface structure consisting of a cytoplasmic syncytium attached to underlying cell bodies by narrow cytoplasmic connections. The nuclei, ribosomes, endoplasmic reticulum, mitochondria and Golgi apparatus are located in these cell bodies, and their vesicular products -the so-called discoid bodies and multilaminate vesicles- are transported to the tegument syncytium *via* the connections. The apical surface of the tegument is made of normal plasma membrane overlaid by a membrane-like secretion, which has been termed membranocalyx [8, 9]. Together with the gastrodermis, this tegument constitutes a major interface between the parasite and its host, and it is critically involved in the complex host-parasite relationship (nutrient update, excretion, osmoregulation, etc.) [10]. Thus, the identification and

characterization of schistosome tegumental molecules is essential for a better understanding of the host-parasite relationship and for defining novel immunological, pharmacological and diagnostic targets. Accordingly, many investigations carried out over the last five decades have focused on the identification and characterization of the schistosome molecules expressed on their tegument and surface membranes [9].

Major advances in the identification of proteins from the schistosome tegument have been made in the last decade as a consequence of the development of proteomics and the sequencing of the *S. mansoni* and *S. japonicum* genomes and transcriptomes (www.schistoDB.org) [11, 12]. These studies have involved the study by proteomics of the whole or part of the tegument after fractionation with different techniques [9, 13-20].

Since the accessibility of a protein in the tegument is an important issue when selecting target molecules for vaccines and drugs, studies aimed at identifying the proteins exposed on the worm surface are particularly interesting [15, 17-19]. Two type of experimental approach have been used to achieve this: (i) The *in vitro* biotinylation of live worms with impermeant biotin reagents to label proteins with amine groups exposed either at lysine residues or at the N-terminus, followed by the purification of the biotinylated proteins and their analysis by mass spectrometry for identification [15, 18]; (ii) Enzymatic shaving by incubation of intact worms with hydrolytic enzymes, trypsin or phosphatidyl-inositol phospholipase C (PiPLC) to release peptides from the surface-exposed proteins and peptide analysis by LC-MS/MS [17, 19].

All these studies have illustrated the dynamic nature of the schistosomal tegument and have provided invaluable information about the repertoire of tegumental molecules that are expressed on the tegument surface and their relative locations at the plasma membrane and membranocalyx. In such studies, besides several proteins of host

origin (immunoglobulins, complement factors and the CD44 and CD90 host cell surface proteins), it has been also identified some schistosome transporters, enzymes and structural membrane proteins, and a variable range of cytosolic and cytoskeletal proteins, whose presence on the surface has been attributed by some authors to tegument damage and the subsequent release of parasite inner cytosolic or structural molecules, and which are interpreted as "false" identifications from the tegument surface [18, 20, 21]. It is known that one of the major limitations of *in vitro* work with schistosomes is the fragility of the worms. Thus, even though the worms are handled with great care it cannot be ruled out that some tegumental damage may lead to biotinylation or enzymatic digestion of internal proteins [20].

Regarding the tegument of *S. bovis*, up to date only three studies have attempted to analyse its composition [16, 17, 22]. In the first two studies, two dimensional (2D) proteomic maps of the tegument were constructed, and the more abundant proteins, the antigenic ones and the glycoproteins were identified by mass spectrometry analyses of the corresponding spots sliced from the 2D gels [16, 22]. The third work, aimed at identifying the proteins expressed on the outermost layers of the tegument structure of adult worms, was the first to apply the enzymatic shaving technique in a schistosome species. Male and female worms were separately subjected to soft trypsin digestion and a wide range of tegumental proteins were identified - most of them for the first time in *S. bovis*-, some differences being observed in the tegumental protein repertoire of both sexes [17].

The aim of the present work was to perform an *in vivo* identification of the host-exposed tegumental proteins from *S. bovis* adult worms. To accomplish this, we used a method that permits the labelling of the surface of the worms inside the blood vasculature and that is based on the vascular perfusion of mice infected with *S. bovis*

with a biotin ester reagent. In this way, the proteins of the tegument surface of the worm entering into contact with the perfusion fluids became biotinylated and were efficiently purified by streptavidin affinity chromatography. Trypsin digestion of the biotinylated proteins and subsequent liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS) resulted in the identification on the *S. bovis* tegument of 80 parasite proteins and 28 host proteins.

2. Material and methods

2.1. Experimental animals and parasites

Thirty-five NMRI mice weighing 30-35 g (Harlam Laboratories Models, S.L.) were used. All mice were infected with 200 *S. bovis* cercariae by the procedure of tail immersion for 60 min. Four months later, when the infections were fully established, twenty animals were perfused with sulfo-NHS-LC-biotin (Thermo Fisher Scientific) as described below. After perfusion, the portal vein was dissected and the live, mobile worms found inside the hepatic-portal system were recovered. In parallel, the remaining 15 *S. bovis*-infected mice, which had not been perfused, were sacrificed and the adult worms collected.

For perfusion and sacrifice, all mice were anesthetized with a combined subcutaneous injection of 200 mg/kg ketamine (Imalgene 1000, Merial) and 20 mg/kg xylazine (Xilagesic 2%, Laboratorios Calier, S.A.)

All animal manipulations were done according to the rules from the ethical and animal welfare Committee from the Institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. Vascular perfusion procedure of infected mice for in vivo *biotinylation of S. bovis adult worms*

We followed the same procedure as that described by Roesli et al. [23]. Briefly, perfusion was performed from the aorta through the hepatic portal system. To do this, a perfusion needle (24-gauge catheter, BD Insyte) was inserted into the aortic artery at the exit from the left ventricle, through which the solutions were administered, and another catheter was inserted at the posterior end of the portal vein to allow outflow of the perfusion solutions. First, blood components were washed away with pre-warmed PBS (38ºC) supplemented with 10% dextran 40 (Sigma) as a plasma expander for 10 min. Immediately afterwards, the mice were perfused with 10 ml of biotinylation solution at a flow rate of 1 ml/min. The perfusion solution contained 1 mg/ml of sulfo-NHS-LC-biotin (Thermo Fisher Scientific) in PBS (pH 7.4, 38 ºC) and 10% of dextran 40. To prevent hydrolysis of the sulfo-NHS-LC-biotin, the reagent was dissolved immediately before the beginning of the *in vivo* perfusion. Subsequently, to neutralize unreacted biotinylation reagent the mice were perfused for 10 min with 50 mM Tris-HCl in PBS and 10% dextran 40. All solutions were perfused using a peristaltic pump and the flow rate was tuned so as not to exceed a counter pressure of 100 mmHg.

Throughout the operation, the mice remained fixed to a solid surface, which was slightly inclined to help the drainage of liquid excess, and were kept warm (38ºC) with the aid of a lamp.

After vascular perfusion, the adult worms inside the portal system were recovered. The worms were rinsed briefly in PBS and observed to confirm that they were live and mobile. Then, they were either freshly snap-frozen for the preparation of protein extract or fixed for 24 hours in 4% saline formaldehyde for immunofluorescence analysis.

2.3. Preparation of protein extracts for proteomic analysis

We followed the same procedure as that described by Roesli et al. [23] with some modifications.

Two different batches of worms were processed to collect protein extracts: Sb0 (control batch), consisting of 50 non-biotinylated adult worms recovered from the 15 non-perfused mice, and Sb1, consisting of 50 biotinylated adult worms recovered from the 20 mice perfused with biotin.

Frozen specimens from each batch were resuspended in 40 µl of lysis buffer per mg of tissue (2% SDS, 50 mM Tris, 10 mM EDTA, complete EDTA-free proteinase inhibitor cocktail (Roche Diagnostics) in PBS, pH 6.8.). The samples were homogenized six times -2 min each at full power- using an Ultra-Turrax T10 disperser (IKA-Werke). The homogenates were sonicated for 3 min, incubated at 95 ºC for 20 min, and centrifuged at 17,000 g for 20 min. Then, to eliminate as much SDS as possible and to avoid interference in biotin-streptavidin binding, the supernatant was dialyzed with PBS for 24 hours. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Thermo Fisher Scientific).

2.4. Purification of biotinylated proteins and on-resin tryptic digestion

Biotinylated proteins from the Sb1 and Sb0 protein extracts were purified using a streptavidin-sepharose resin (GE Healthcare). The slurry resin was washed three times in a buffer containing 1% Nonidet P-40 and 0.1% SDS in PBS (pH 7.4). 4.4 mg of total protein extract was added to 190 µl of resin and biotinylated proteins were captured for 2 h at room temperature in a revolving mixer at low rotation speed. The unbound proteins were eliminated by washing 3 times with the above-mentioned buffer, followed

by centrifugation at 470 g. Then, the resin samples containing the bound biotinylated proteins were washed five times with 50 mM NH_4HCO_3. Cysteine residues of proteins were reduced by 2 mM Dithiothreitol (DTT) in 50 mM NH_4HCO_3 at 60ºC for 20 min and sulfhydryl groups were alkylated with 5 mM Iodoacetamide (IAM) in 50 mM NH_4HCO_3 in the dark at room temperature for 30 min. Following this, excess IAM was neutralized with 10 mM DTT for 30 min at room temperature. Then, proteins were digested overnight at 37 ºC with 1 µg of sequencing grade modified trypsin (Promega) in 50 mM NH_4HCO_3. The reaction was stopped with trifluoroacetic acid (TFA) at a final concentration of 0.1%. The final peptide mixture was concentrated in a speed vacuum at a final volume of 50 µl and was cleaned with a ZipTipSCX Pipette Tip (Millipore). The concentration of the peptides was estimated by absorbance at 280 nm in a nanoDrop instrument. Two aliquots of each one of the two tryptic peptide samples (from Sb0 and Sb1) were then analysed by liquid chromatography and tandem mass spectrometry.

2.5. Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

The proteomic analysis was carried out at the SCSIE_University of Valencia Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform.

LC-MS/MS was performed as described previously by De la Torre-Escudero et al. [24] with some modifications. Briefly, two aliquots of each tryptic peptide samples (Sb0 and Sb1) corresponding to 2 µg of digested peptides was loaded onto a trap column (NanoLC Column, 3µ C18-CL, 75 µm×15 cm, Eksiger) and desalted with 0.1% TFA at 3µl/min for 5 min. The peptides were then loaded onto an analytical column (LC Column, 3µ C18-CL, 75 µm×25 cm, Eksiger) equilibrated with 5% acetonitrile and 0.1% formic acid. Elution was carried out with a linear gradient of 5%- 35% acetonitrile

in 0.1% formic acid over 120 min at a flow rate of 300 nl/min. The peptides eluted were analyzed with a nanoESI Q-TOF mass spectrometer (5600 TripleTOF, ABSciex) in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350-1250 m/z was performed, followed by 0.05-s product ion scans from 100-1500 m/z on the 50 most intense 2-5 charged ions.

2.6. Database search and protein identification

Database searches were carried out in the NCBInr Metazoa protein database (2841375 sequences) in a custom-made Trematode EST database (2471628 sequences) and in the NCBInr protein database (22470027 sequences) using MASCOT v2.2 (Matrix Science) and ProteinPilot v4.0 (AB Sciex) search engines.

For Mascot searches, the peak lists were generated directly from QSTAR wiff files by Mascot Daemon v 2.2.2 (Matrix Science) with Sciex Analyst import filter options using the default parameters. The NCBInr Metazoa and Trematoda EST database were searched using the following parameters: tryptic specificity, allowing two missed cleavages and tolerance in the mass measurement of 50 ppm in MS mode and 0.5 Da for MS/MS ions. The carbamidomethylation of Cys was set as a fixed modification, and Met oxidation and Asn/Gln deamidation were set as variable modifications. The significance threshold was set at 0.05. The criterion for accepting protein identification was that the identification had to contain two or more unique peptides with a score greater than the identity threshold supplied by the Mascot server.

For ProteinPilot searches, default parameters were used to generate the peak list directly from 5600 TripleTof wiff files of Sb0 and Sb1 samples. The Paragon algorithm of ProteinPilot was used to search the NCBInr protein database with the following parameters: trypsin specificity, cys-alkylation, taxonomy restriction to yeast and the

search effort set to rapid. To avoid using the same spectral evidence in more than one protein, the proteins identified were grouped based on MS/MS spectra, using the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra were grouped, regardless of the peptide sequence assigned. The protein within each group able to explain more spectral data with confidence is shown as the primary protein of the group. A protein was considered to be identified when two or more peptides, each with a confidence > 99%, were positively matched in the database.

The relative abundance of a protein in the sample was quantified using the protein abundance index (PAI), which is defined as the number of observed peptides in the experiment divided by the number of observable tryptic peptides for each protein within a given mass range of the mass spectrometer employed [25]. PAI was modified exponentially to give emPAI, the exponential form of PAI minus one, which is directly proportional to the protein content in a sample [26].

Functional and cellular locations of the proteins were assigned according to the SwissProt/UniProt databases (http://www.uniprot.org/), the Gene Ontology database (http://amigo.geneontology.org), the National Center for Biotechnology Information database (NCBI: http://www.ncbi.nlm.nih.gov/sites/entrez) and the literature. In addition, the following predictions were also performed: presence of signal peptide using SignalP 3.0 [27] at http://www.cbs.dtu.dk/services/SignalP; presence of transmembrane helices using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0, and presence of glycosyl-phosphatidyl anchor sites using the PredGPI prediction server (http://gpcr.biocomp.unibo.it/predgpi/index.htm) [38].

In the Results section, redundant identifications are eliminated from the lists of identified proteins, in each case choosing the protein hit with the highest score and adding the emPAIs of the redundant identifications.

2.7. Immunofluorescence analysis to confirm biotinylation of adult worms.

To verify the surface biotinylation of worms and estimate the extent of biotin incorporation to the tegument, adult worms recovered from mice were fixed in 4% formaldehyde in PBS for 24 h and then dehydrated and embedded in paraffin following standard protocols. Microtome-cut 5 μm sections were placed on microscope slides, deparaffinised in xylene, and rehydrated.

In these sections the biotin tag was detected by fluorescence, using a streptavidin-fluorescein isothiocyanate (FITC) probe. Sections were blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST) for 1 h at 37 ºC, and incubated with streptavidin-FITC (Sigma) diluted 1/500 in blocking buffer for 1 h at 37 ºC. Sections were washed five times with PBST and mounted in Prolong Gold antifade reagent (Invitrogen) with 4', 6-diamidino-2-phenylindole (DAPI, Sigma). As a control, non-biotinylated worms were prepared and labelled as described. All incubations were performed in a humid chamber.

2.8. SDS-PAGE and western blotting

The composition of the Sb0 and Sb1 protein extracts from adult worms, the biotinylated protein and the efficiency of purification were checked by SDS-PAGE and western blot with streptavidin-HRP. Samples of 10 μg of Sb0 and Sb1 protein extracts, the supernatant after capture, and resin-bound proteins were subjected to electrophoresis in 10% acrylamide gels. After running at 60 V, the gels were either silver-stained or

electrotransferred onto nitrocellulose membranes at 400 mA for 90 min. The membranes were blocked in 2% BSA, washed with PBST, and then incubated with streptavidin-HRP (Sigma) diluted 1/5,000 for 1h at room temperature. Immunoblots were developed with the Immun-Star HRP Chemiluminescent Kit (Bio-Rad) and images were digitalized with the ChemiDoc MP Imagen System (Bio-Rad).

3. Results

3.1. Biotinylation of adult worms after vascular perfusion of the infected mice

S. bovis adult worms recovered from mice perfused *in vivo* with the biotin reagent were subjected to immunofluorescence microscopy to verify the biotin labelling efficiency and the biotin distribution pattern.

The images in Fig. 1 show adult worm sections after streptavidin-FITC incubation and DAPI staining. A strong uniform green signal can be observed throughout the tegument surface without any green signal inside the tegumental cytoplasm or in inner tissues, suggesting selective labelling of the worm surface. Fig. 1C also shows some biotinylation of the components of the ventral sucker. No biotin-labelling of the gastrodermis was observed in any of the sections.

In the section of adult worms collected from the non-perfused mice, no type of fluorescence was observed and only the staining of the cell nuclei with DAPI was visualized (not shown).

3.2. Protein composition of extracts and purification efficiency of biotinylated proteins.

As shown in Fig.2, after silver staining of the SDS-PAGE gels the total protein extracts of adult worms (Sb0 and Sb1) showed a similar band pattern, displaying many components. In the western blot extract of the Sb0 protein extract and of the

corresponding resin sample an intense band of 120 kDa was visible. Additionally, in the lane carrying this resin sample it was possible to observe, although much more faintly, another band of some 80 kDa (Fig. 2A). These sizes are consistent with the molecular weight of the enzymes pyruvate carboxylase (129,684 kDa) and propionyl CoA carboxylase (79,940), which contain several biotin-binding sites and were abundantly identified in the analysis by LC-MS/MS of this sample (next section).

The western blot of the Sb1 extract and of the resin containing the biotinylated proteins (R1) also revealed, apart from the band of 130 kDa also present in the Sb0 extract, several components that had a broad range of molecular weights (20-130 kDa) (Fig. 2B).

As can be observed in Fig 2, the biotinylated proteins were efficiently captured on the streptavidin-sepharose resin, no biotinylated compound being observed in the lanes corresponding to the supernatants after capture (U1).

3.3. Proteins identified

The identified proteins from samples Sb0 and Sb1 shown in the results are the sum of the identifications performed with the Mascot and ProteinPilot algorithms in the NCBInr metazoa protein, NCBInr protein and Trematoda EST databases. Redundant identifications and contaminant proteins such as keratins, proteins similar to keratins, trypsin and streptavidin from sepharose beads are excluded from the lists of identified proteins (Tables 1 and 2, Supplementary Tables 1 and 2).

The Sb0 sample was included in this study as a control to identify the proteins that might have been non-specifically retained in the resin (Supplementary Table 1). Twenty-five non-redundant proteins were identified in sample Sb0: 6 intracellular parasite proteins that contained several biotin-binding sites and were biotinylated *per se*

such that they bound to the streptavidin resin; 8 proteins from *S. bovis* origin (actin, collagen alpha-1(V), gynecophoral canal protein, LdL receptor, myosin heavy chain, and three hypothetical proteins) among which, and according to the literature, only actin has been properly identified on the tegument surface; 6 proteins (desmocollin, desmoglein, desmoplakin, filaggrin, hornerin, plakoglobin) that form part of the human epidermis and are linked to the keratinization of epithelial tissues. These were accordingly considered as contaminations produced during the handling and preparation of the samples. Finally, host albumin and 4 proteins of unknown origin (matrix protein 2-1, rubber elongation factor protein, the transcription antitermination protein nusg, URT reporter protein) were also identified. These last four proteins were only identified by Protein Pilot searching in the NCBInr database (Supplementary Table 1).

All the proteins identified in the Sb0 sample were also detected in the Sb1 sample and were considered as contaminants and/or non-specifically bound to the streptavidin resin. Accordingly, all of them except actin were excluded from the final list of identifications for Sb1, described below (Tables 1, 2, 3 and Supplementary Table 2).

As a result, a total of 117 non-redundant proteins were identified in the biotinylated worms: 80 proteins were from the parasite, 28 from the host and 9 were of an undetermined origin. The parasite proteins identified were grouped according to their predicted subcellular location (Table 1, Figure 3).

Among these 80 schistosome proteins, 20 were classified as cytosolic proteins, 13 as cytoskeletal proteins, 22 as membrane- and tegument-associated proteins and 25 were hypothetical, uncharacterized, or proteins of unknown ontology. The group of cytosolic proteins contained the most abundant proteins, elongation factor-1 (emPAI= 4.42) and glutathione-S transferase (emPAI= 3.69) (Table 1, Fig. 3A).

Among the 13 cytoskeletal proteins, the most abundant by far was actin, showing a very high emPAI (56.53), higher indeed than the sum of the emPAIs of all the other protein identified in sample Sb1. For this reason, and to avoid masking the relative abundance of the other groups of proteins represented in Fig. 3A, the actin emPAI was not included in the global emPAI of the cytoskeletal protein group.

The group of membrane- and tegument-associated proteins was the most numerous and abundant. The analysis of their sequences predicted the presence of 1 to 12 transmembrane domains for 13 of them, and the presence of both a GPI anchor and signal peptide for the 200 kDa surface tegumental protein. The remaining eigth tegument-associated proteins, namely, Sm20.0, Sm20.8, 22.6 tegument antigen, annexin, calpain, enolase, glyceraldehyde 3-phosphate dehydrogenase and heat shock protein 70 lacked transmembrane domains, GPI anchors and signal peptide.

Twenty-eight proteins from the host were also identified in the tegumental surface of *S. bovis* (Table 2, Fig. 3B). These were mainly blood components involved in coagulation and transport, such as apolipoprotein, fibrinogen, haemoglobin, transferrin and serine protease inhibitor A3K, being haemoglobin and fibrinogen particularly abundant; immune response components (several immunoglobulins, C3 and C4 complement components and bone marrow proteoglycan precursor), and vascular endothelium and leukocyte surface proteins (Table 2, Fig. 3B).

Finally, in the biotinylated worms, another 9 proteins were identified whose origin could not be assigned either to the parasite or to the host. These proteins, which are not included in the above-mentioned groups, were 3 uncharacterized proteins, 2 stress-response proteins, 2 histones and 2 epidermal proteins from *Homo sapiens* (dermicin and suprabasin), which could have been contaminants that were added during the handling of the samples (Supplementary Table 2).

3.4. Comparison of the tegumental proteins identified with other proteomic studies

The parasite proteins identified in this study were compared to those found in other proteomic studies of the tegument surface of schistosomes

As can be observed in Table 3, of the 20 cytosolic proteins identified, up to 14 (70%) have been identified on the schistosome tegument. These proteins were identified by enzymatic shaving of the *S. mansoni* and/or *S. bovis* worm surface, and 5 of them were also identified by biotinylation on the surface of *S. mansoni* and/or *S. japonicum*.

Similarly, of the 13 cytoskeletal proteins identified in *S. bovis* in this work, up to 8 (61.5%) have been identified on the schistosome tegument, mostly by enzymatic shaving.

Regarding the 22 membrane and tegument-associated proteins, up to 19 (86.4%) have been already identified on the schistosome tegument surface. In contrast to the cytosolic proteins, most of these membrane- and tegument-associated proteins had been identified on the *S. mansoni* and/or *S. japonicum* tegument by *in vitro* biotinylation of their surface.

Finally, Table 3 also shows that 40.0%, 53.8% and 36.4%, respectively, of the cytosolic, cytoskeletal and membrane proteins identified in the current work, had been previously detected in the proteomic study of the *S. bovis* tegument by Perez-Sánchez et al. [17] using soft trypsin shaving of the worm suface.

4. Discussion

As stated in the Introduction, the purpose of this work was to identify the proteins expressed on the surface of the tegument of *S. bovis* adult worms after selective labelling *in vivo*. The biotinylation of worms inside the blood vessels of infected mice

by vascular perfusion with biotin avoids having to handle the worms *in vitro*, which could involve possible tegument damage and in turn could make the results difficult to interpret.

The method of vascular perfusion *in vivo* with sulfo-NHS-LC-biotin has been used in several disease models in order to establish an atlas of vascular proteins in health and disease [29-33]. More recently, we applied this methodology to study the proteome of the endothelial surface of the mouse portal vein, and we identified some of the changes induced in it after infection by *S. bovis* [24]. The present work is the first in which vascular perfusion has been used to investigate, *in vivo*, the proteins exposed by an intravascular pathogen on its surface to the host.

The isolation of biotinylated proteins from adult worms by streptavidin-sepharose resin followed by on-resin tryptic digestion and LC-MS/MS analysis led to the identification of 80 parasite proteins and 28 host proteins. This number of proteins is higher than what would be expected taking into account the studies conducted on the surface of the tegument of *S. mansoni* and *S. japonicum* after *in vitro* labelling with biotin or enzymatic shaving [15, 18, 19]. In those works, the authors identified several membrane- and tegument-associated proteins as well as a number of cytosolic and cytoskeletal proteins. They ruled out the accessibility of these intracellular proteins on the tegument surface and attributed the identification of cytosolic and cytoskeletal proteins to damage caused to the tegument during the *in vitro* handling of the worms [20]. Those authors also suggested that some of these intracellular proteins might have been pulled down by protein-protein interactions with *bona fide* membrane proteins during incubation of the solubilised proteins with streptavidin [18].

In the current study, we have also identified a range of cytosolic and cytoskeletal proteins in the biotinylated fraction of the tegument of *S. bovis*. However, we believe it

is fairly unlikely that the vascular perfusion with biotin performed in the mice could have damaged the worms inside the vessels resulting in the biotinylation of proteins of the tegumental cytoplasm. Worms were collected from the vascular bed alive and mobile and, additionally, in the histological sections we observed that the labelling was limited to the tegumental surface and suckers. On the other hand, we also find quite unlikely that non-labeled intracellular proteins could have been co-purified together with the biotinylated surface proteins since the treatment of the worms with 2% SDS prior to the streptavidin chromatography would have disrupted the protein-protein interactions involved.

Different studies have shown that the rate of membranocalyx turnover appears to vary depending on conditions in the external environment of the worms [9]. In addition, the maintenance of the apical membrane and membranocalyx is mediated by the fusion of membranous vesicles -originating from the tegumentary cell bodies- with the apical membrane and the subsequent release of their contents into the membranocalyx [18]. Then, it could be speculated that if the membranocalyx turnover of the worms is higher *in vivo* than *in vitro,* this would result in a greater number of intracellular tegumental molecules transiently exposed to the host and therefore to biotin during perfusion.

Additionally, it is also possible that some of the proteins identified in the biotinylated fraction of the tegument could form part of exosome-like vesicles, which would be released by the schistosome tegument in a similar way to that described for the schistosome gut, where they are apparently released by the epithelium to the lumen [34]. The presence of vesicles in the tegument of trematodes had been already described by Threadgold in 1963 [35]. In a study addressing the ultrastructure of the tegument of *Fasciola hepatica* this author remarked the existence of "a great number of small membrane-limited vacuoles and vesicles". Recently, in a revealing study, Marcilla et al.

[36] used electron microscopy to identify the presence of exosome-like vesicles on the tegument surface of *Echinostoma caproni* and they identified the protein content of these purified extracellular vesicles from *E. caproni* and *F. hepatica*. They suggested the possibility that the release of exosomes by the parasite, as well as constituting a strategy to manipulate the host cell biology and the immune response, could account for the presence of cytosolic and cytoskeletal proteins in the schistosome worm vomitus and in the secretome of parasitic trematodes [34, 36]. The hypothesis on the release of exosome-like vesicles has been also proposed by other authors to explain the intriguing presence of predicted cytoplasmic proteins in the "surfomes" (surface-accessible molecules of infectious or parasitic organisms) [37]. Evidently, further specific studies should be conducted, similar to those performed with *E. caproni*, to confirm the release of this type of vesicle from the schistosome tegument.

Regarding the repertoire of tegument proteins identified in the current work, only 43% of them had also been found by Perez-Sánchez et al. [17] after soft trypsin shaving of male and female *S. bovis* worms, mostly of them being cytosolic/cytoskeletal and only 8 membrane- and tegument-associated proteins. This low correspondence could be explained by keeping in mind that the proteome analyzed by Pérez-Sánchez et al. [17] comprised not only the surface but also the outermost layers of the tegument structure, which were exposed to trypsin as a result of the worm fixation with methanol. Therefore, it can be argued that perhaps in the referred study a range of low abundance surface-exposed proteins could have been not detected owing to the presence of some more abundant internal proteins.

Of the 20 cytoplasmic proteins identified in *S. bovis*, 14 have been identified in earlier studies addressing the schistosome tegument. By contrast, this is the first time that Clathrin, Rabs proteins and glucose-regulated protein GRP78 have been identified

in the schistosome tegument. Clathrin and Rabs proteins are molecules related to traffic pathways and vesicular transport [38, 39]. These activities suggest that these proteins could be involved in the vesicle migration from the cytons into the tegumental cytoplasm and fusion at the apical membrane [9]. The glucose-regulated protein GRP78 is a stress protein belonging to the HSP70 multigene family that shows 76% identity to its homologous protein identified in the endothelial vascular surface of the portal vein of *S. bovis*-infected mice [24]. The expression of this protein in the vascular endothelium has an inhibitory effect on coagulation through the regulation of tissue factor [40], such that it is possible that the GRP78 expressed on the *S. bovis* tegument might have a similar function. It has been shown that the parasite has several molecules with anticoagulant, profibrinolytic and vasodilator activity [5-7, 41].

Regarding the cytoskeletal proteins, besides actin, which is known to be present in the spines and tubercles of tegument, we identified several proteins with motor activity. Some of them have never been found in the schistosome tegument, and for others, such as paramyosin and myosin, their localization on the surface of tegument, outside the schistosome muscle, is controversial [9]. These two molecules have shown experimental promise as vaccine candidates, which suggests that they should be accessible, at least transiently, to the host immune system components [42, 43].

With respect the tegument –associated proteins, it is worth noting that annexin, enolase and glyceraldehide 3-phosphate dehydrogenase had been previously detected on the tegument surface of *S. bovis* adult worms by immunofluorescence assays [6, 7, 17].

We also identified many (22) hypothetical or uncharacterized parasite proteins, which is higher than the figures found in another similar study in *S. mansoni* [15], perhaps because of the better sensitivity of our method of analysis as compared with the polyacrylamide gels and in-gel tryptic digest used in that study. Notwithstanding, this

result is not very surprising if it is considered that approximately 40% of the schistosome genome is composed of hypothetical proteins of unknown function [44]. As indicated by van Balkom et al. [13], these proteins unique to the parasite may perform roles that are specific to the schistosome tegument, with their unique sequence and structure making them excellent targets for anti-schistosome drugs.

In this study, in the *S. bovis* tegument surface we failed to detect some proteins, such as alkaline phosphatase, tetraspanin and Sm29, that have previously been identified on the surface of *S. mansoni* and *S. japonicum* by proteomic and non-proteomic methods [9, 18, 20]. Perhaps the level of expression of these molecules in the *S. bovis* tegument might be too low to be detected, being masked by more abundant proteins such as actin. Another possibility is that our results could be unveiling molecular differences in the tegument between schistosome species. Similarly, Mulvenna et al. [18] were unable to find the homologue of the Sm29 protein (Sj29) in the biotinylated fraction of *S. japonicum*, suggesting a weaker association with the tegument than that observed for Sm29 in *S. mansoni*. Regarding tetraspanins, a recent study on the TSP-23 from 10 schistosome species revealed a great diversity in the sequences of the external loops of these molecules, being the TSP-23 of *S. bovis* the most distant as compared to those of *S. mansoni* and *S. japonicum* [45]. Therefore it could be speculated that perhaps the external loops of the *S. bovis* TSP-23 lack biotin binding motifs thus hindering its purification and identification. It could be also possible that, as suggested before, the level of expression of TSPs in the *S. bovis* tegument might be too low to be detected or even that these proteins should not be expressed on the tegument surface, as has been observed for SmTSP-2 [46].

Furthermore, we did not detect any enzyme-released proteins in the alimentary tract, confirming the lack of biotin labelling of the parasite gastrodermis.

A number of host proteins have also been identified on the *S. bovis* tegument (Table 2). As observed for *S. mansoni* and *S. japonicum* [15, 18, 19], *S. bovis* adult worms were coated with several host immunoglobulins, CD44 and CD47 surface cell proteins, haemoglobin, and C3 and C4 complement components. In addition, on the *S. bovis* tegument we found some proteins that had never been identified on the schistosome surface, such as several blood components (Apolipoprotein, transferrin, a serine protease inhibitor and fibrinogen in great abundance) and proteins from the host endothelial vascular surface (basigin, dipeptidil peptidase, and vitronectin). The presence of these last proteins on the surface of *S. bovis* reflects the intimate contact of the parasite with endothelia during intravascular movement [24]. We believe that one reason for failing to identify these proteins at the surface of the tegument in the *in vitro* studies would be that they may be easily lost during the washings and manipulations to which the parasites were subjected.

Having in mind the biology of the schistosomes and the strategies they have developed to avoid host coagulation and promote vasodilatation, the presence of pro-coagulant molecules, such as fibrinogen, on their surface could be controversial (5-7, 41). By one hand, it could be hypothesized some kind of profibrinolytic function for fibrinogen as a cofactor for the plasminogen/receptor complex, as has been described that happens in some bacterial infections. In group A streptococci, it has been demonstrated that one pathway to gain fibrinolytic activity is streptokinase/plasminogen complex with fibrinogen as cofactor [47]. On the other hand, it could be possible that the presence of fibrinogen, and possibly of other host proteins, on the parasite surface lacks a functional significance for schistosome but is merely a consequence of the chemical and biophysical nature of its outer surface [9].

It is also worth mentioning that we failed to identify host plasminogen on the tegument despite the fact that this is an abundant plasma protein and that *S. bovis* can bind plasminogen on its surface [5]. Similarly, in other study in which we applied the same methodology to analyze the proteome of the endothelial surface of the portal vein of naïve mice and mice infected with *S. bovis* we also failed to detect plasminogen in spite of the presence of plasminogen receptors on the endothelial surface [24]. As a whole, these results seem to suggest that the method used in both studies would not be appropriate to capture and identify this protein.

In conclusion, the experimental approach used in this work is a reliable method to selectively investigate the surface of this parasite preventing the handling of worms *in vitro* and so eventually tegument damages, and providing valuable information about the exposed protein repertoire of the tegument of *S. bovis* in the environmental conditions that the parasite faces inside the blood vessels.

Acknowledgments

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Figure captions

Figure 1. Biotinylation of the adult worms recovered from mice perfused with biotin. Paraffin sections of the adult worms and fluorescence staining with streptavidin-FITC (green) and DAPI (blue). Panels: A, 200x magnification; B and C, 400x magnification and D, 1000x magnification.

Figure 2. Analysis of the extracted and purified proteins from non-biotinylated (A) and biotinylated (B) *Schistosoma bovis* adult worms. Silver-stained SDS-PAGE and western blot with streptavidin-HRP: whole protein extracts of non-biotinylated (Sb0) and biotinylated (Sb1) adult worms; unbound proteins after incubation with the streptavidin-sepharose (U0, U1); and proteins bound to the resin (R0 and R1).

Figure 3. Number of proteins and total emPAI values of parasite and host proteins identified on the tegument surface of *Schistosoma bovis* adult worms. Parasite proteins are grouped according to their cellular location and host proteins according to their function. The emPAI value of each group is the sum of the emPAI of each protein in the group and accounts for the relative abundance of protein group in the sample.

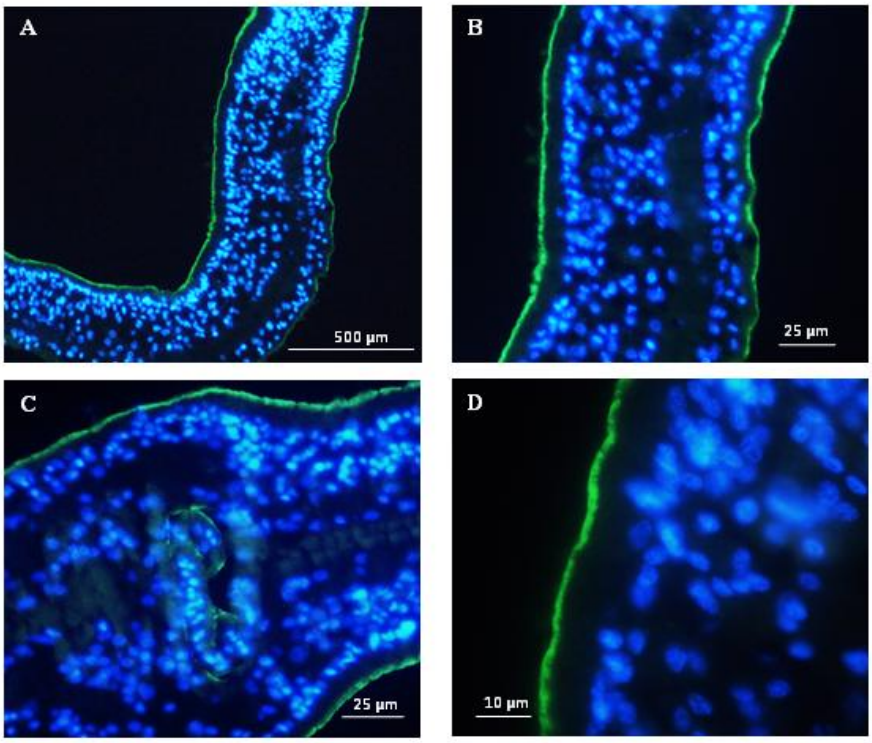


Figure 1.

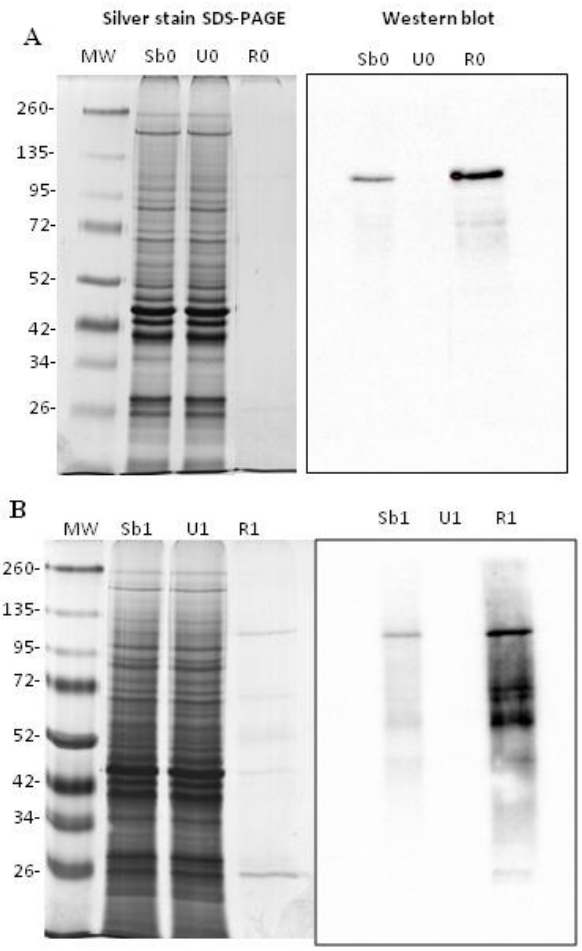


Figure 2.

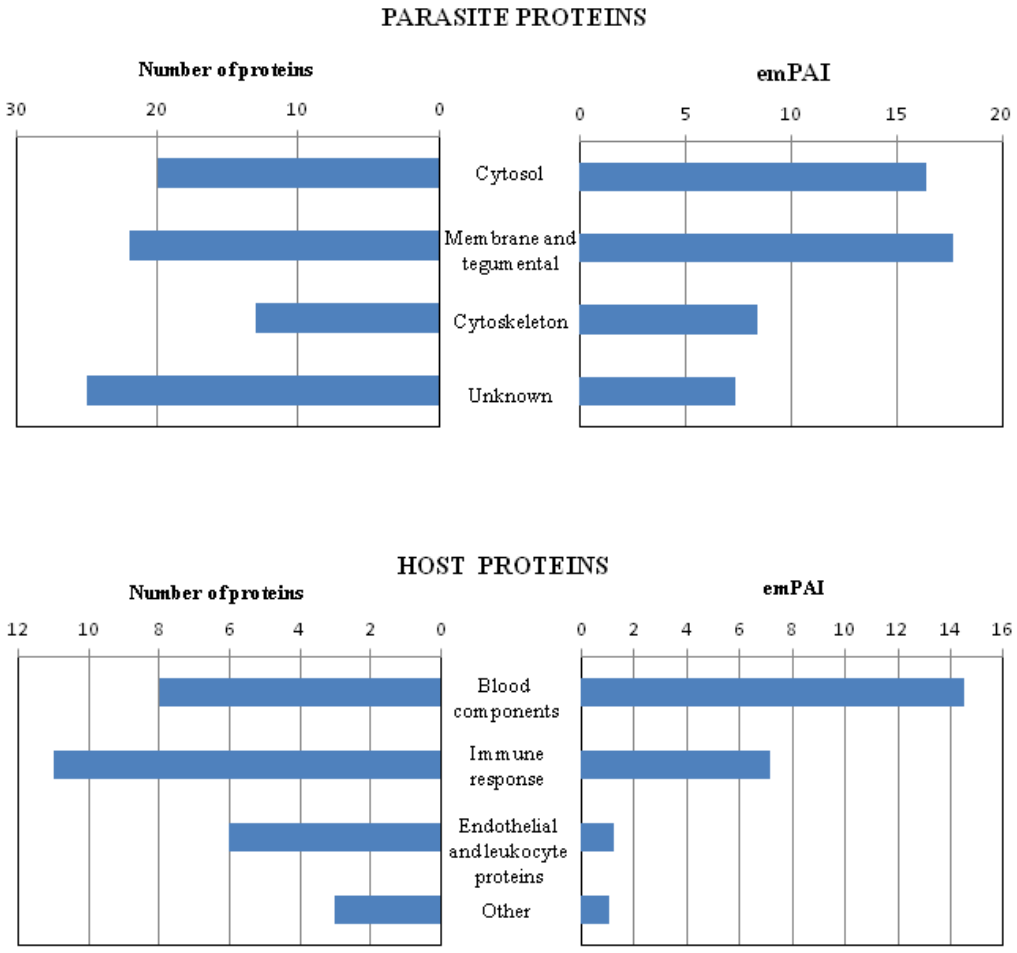
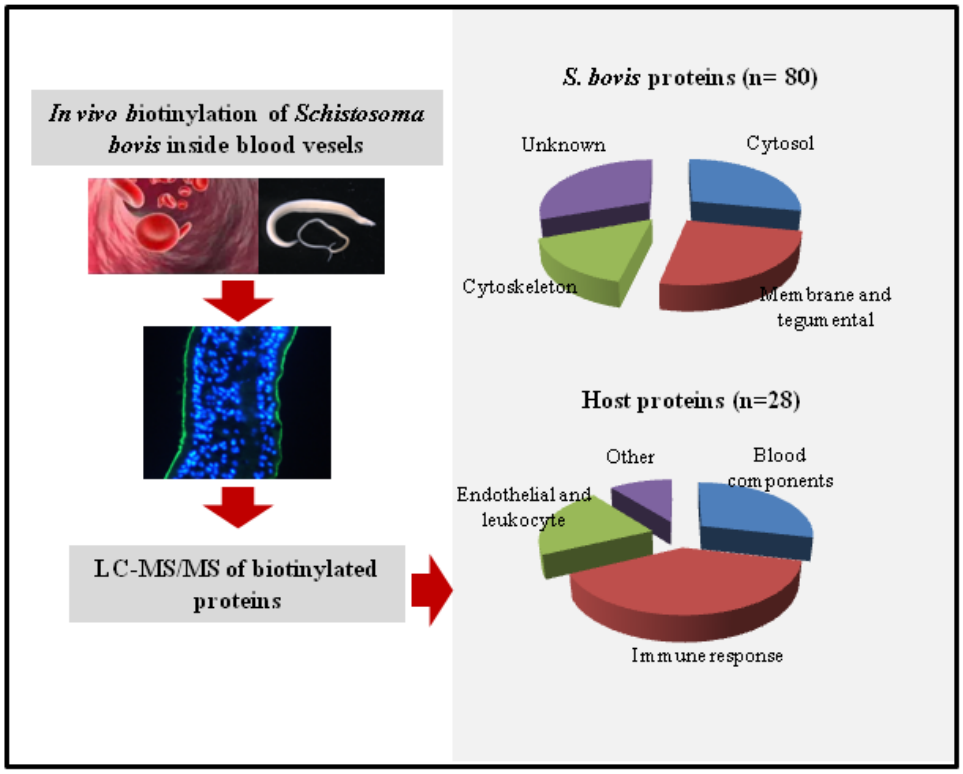


Figure 3.



Graphical Abstract

Table 1. Non-redundant parasite proteins identified on the tegumental surface of adult *Schistosoma bovis* worms. The accession number (NCBI) indicates non-redundant identifications by Mascot and ProteinPilot in the NCBI database; the gene index number corresponds to identifications by Mascot in the EST trematode database.

Accession number (NCBI)	Gene index number	Protein name	Species	Score (Mascot/ Pilot) [a]	Predicted TMs [b]	GPI [c]	Signal peptide	PN (Mascot/ Pilot) [d]	em PAI
Cytosolic proteins (n= 20)									
gi|47933556	Smp_009760	14-3-3 zeta isoform	*S. bovis*	185/10.5	0	0	no	4/5	0.73
	Smp_034840	14-3-3 protein epsilon	*S. mansoni*	72	0	0	no	2	0.6
gi|256082104		Clathrin heavy chain	*S. mansoni*	71	0	0	no	3	0.06
gi|256070913		Endophilin B1	*S. mansoni*	97/4.0	0	0	no	2/2	0.25
gi|269117259	Smp_099870	Elongation factor-1 alpha	*Euptera elabontas*	115/10.0	0	0	no	3/5	4.42
gi|47933560	Smp_095360	Fatty acid binding protein 15	*S. bovis*	209/6.4	0	0	no	4	1.28
gi|186462281	Smp_042160	Fructose bisphosphate aldolase	*S. bovis*	323/15.1	0	0	no	8/7	1.05
gi|32481989		Glucose regulated protein GRP78	*Spirometra erinaceieuropaei*	226	0	0	yes	2	0.09
gi|544443	Smp_054160	Glutathione-S transferase 28kda	*S. bovis*	485/10.5	0	0	no	6/4	3.69
gi|360045358	Smp_143840	Glycogen phosphorylase	*S. mansoni*	299/11.2	0	0	no	8/4	0.71
gi|256086226		Guanosine-diphosphatase	*S. mansoni*	4.0	0	0	no	2	-
gi|353230105		Heat shock protein 90	*S. mansoni*	8.74	0	0	0	4	-
gi|256077954		L-lactate dehydrogenase	*S. mansoni*	4.2	0	0	no	2	-
gi|256079925	Smp_049300	Major egg antigen (p40)	*S. mansoni*	552	0	0	no	5/6	1.6
gi|256077952	Smp_038950	Malate dehydrogenase	*S. mansoni*	285	0	0	no	2	0.42
gi|1172460		Phosphoglycerate kinase	*S. mansoni*	124/8.9	0	0	no	3/4	0.24
gi|256088898	Smp_096760	Phosphoglycerate mutase	*S. mansoni*	102/5.9	0	0	no	3/3	0.56
gi|256083139	Smp_065610	Pyruvate kinase	*S. mansoni*	184/12.3	0	0	no	6/6	0.44
gi|256084269	Smp_071630	Rab-2414	*S. mansoni*	111/9.3	0	0	no	3/4	0.69

gi|146741274		Triose phosphate isomerase	*S. haematobium*	90	0	0	no	2	0.25
Cytoskeletal proteins (n= 13)									
gi|226472932	Smp_046600	Actin	*S. japonicum*	1303/28.4	0	0	no	12/13	56.53
gi|256070267		Filamin	*S. mansoni*	74	0	0	no	2	0.02
gi|495668		Fimbrin	*S. mansoni*	188/7.7	0	0	no	3/4	0.14
gi|256070491	Smp_001040	Kinesin heavy chain	*S. mansoni*	67	0	0	no	2	0.14
gi|350646247		Lamin. putative	*S. mansoni*	178/9.3	0	0	no	4/4	0.02
gi|5305329	Smp_045220	Myosin light chain	*S. mansoni*	122	0	0	no	2	0.4
gi|226469350		Myosin regulatory light chain 2	*S. japonicum*	122/6.1	0	0	no	2/3	0.83
gi|146741272	Smp_021920	Paramyosin	*S. haematobium*	344/26.7	0	0	no	9/13	0.57
gi|256078002	Smp_143470	Spectrin beta chain brain 3	*S. mansoni*	119/10.6	0	0	no	7/4	0.1
gi|42559587	Smp_044010	Tropomyosin	*S. haematobium*	138/5.6	0	0	no	4/2	0.47
gi|226478018		Troponin T	*S. japonicum*	82/5.2	0	0	no	2/3	0.29
gi|344295288	Smp_090120	Tubulin alpha-3 chain-like	*Loxodonta africana*	209/12.2	0	0	no	7/6	0.5
gi|30995481	Smp_078040	Tubulin beta	*S. japonicum*	201/13.1	0	0	no	7/5	4.91
Membrane and tegument-associated proteins (n= 22)									
gi|2506253	Smp_005350	20 kDa calcium-binding protein (SM20,0)	*S. mansoni*	124	0	0	no	3	0.68
gi|186462273		22.6kDa tegument-associated antigen	*S. bovis*	118	0	0	no	2	0.31
gi|360043681	Smp_079220	ADPATP carrier protein	*S. mansoni*	339/6.7	2	0	no	3/3	2.99
gi|256085472	Smp_077720	Annexin	*S. mansoni*	71/9.2	0	0	no	2/3	0.17
gi|256071763		Atpase	*S. mansoni*	85	9	0	no	2	0.06
gi|256078530	Smp_042020	ATP-diphosphohydrolase 1	*S. mansoni*	555/11.2	2	0	no	5/5	0.51
gi|3859490	Smp_136710	Calcium atpase 2	*S. mansoni*	435/4.0	8	0	no	11/2	0.7
gi|256075646		Calcium-transporting atpase	*S. mansoni*	18.1	7	0	no	8	-
gi|146741270	Smp_157500	Calpain (Smp_80)	*S. haematobium*	269/15.4	0	0	no	8/7	0.34
gi|256073298	Smp_130280	Cell polarity protein	*S. mansoni*	192	2	0	no	5	0.08
gi|256077282		Dysferlin1	*S. mansoni*	148/11.4	1	0	no	6/5	0.11

gi|186462279	Smp_024110	Enolase	*S. bovis*	319/9.0	0	0	no	7/3	0.84
gi|186462283	Smp_056970	Glyceraldehyde 3-phosphate dehydrogenase	*S. bovis*	497/15.9	0	0	no	11/7	2.98
gi|10168	Smp_106930	Heat shock protein 70	*S. mansoni*	426/13.4	0	0	no	8/6	4.36
gi|353228805		Innexin	*S. mansoni*	5.0	4	0	no	3	-
gi|256081668		Phosphodiesterase	*S. mansoni*	4.0	1	0	no	2	-
gi|350644272		Plasma membrane calcium-transporting atpase	*S. mansoni*	211/9.6	6	0	no	5/5	0.4
	Smp_072290	Rab15 13 10 1 35 5 and	*S. mansoni*	83	1	0	no	2	0.54
gi|15824396		Snak1	*S. mansoni*	363/18.9	8	0	no	8/9	0.95
gi|256072445	Smp_015020	Sodium bicarbonate cotransporter	*S. mansoni*	116	12	0	no	2	0.04
gi|501209	Smp_017730	Surface protein	*S. mansoni*	673/27.0	0	yes	yes	13/13	0.33
	Smp_086530	Tegumental protein Sm 20.8	*S. mansoni*	87	0	0	no	2	0.54
Unknown ontology or uncharacterized (n= 25)									
	Smp_131110	Hypothetical protein	*S. mansoni*	188	8	0	no	5	0.83
	Smp_139100	Hypothetical protein	*S. mansoni*	121	0	0	no	3	0.63
	Smp_111690	Hypothetical protein	*S. mansoni*	118	0	0	no	2	0.35
	Smp_157380	Hypothetical protein	*S. mansoni*	101	0	0	no	2	0.53
	Smp_008110	Hypothetical protein	*S. mansoni*	99	0	0	no	2	0.5
	Smp_107320	Hypothetical protein	*S. mansoni*	94	0	0	no	2	0.32
	Smp_172950	Hypothetical protein	*S. mansoni*	90	0	0	no	2	0.27
gi|256087454		Hypothetical protein	*S. mansoni*	205/11.74	0	0	no	11/4	0.15
gi|256076562		Hypothetical protein	*S. mansoni*	122/7.14	0	0	no	4/3	0.09
gi|256091700		Hypothetical protein	*S. mansoni*	78	0	0	no	3	0.27
gi|256072401		Hypothetical protein	*S. mansoni*	68	1	0	yes	2	0.13
gi|256071927		Hypothetical protein	*S. mansoni*	67	0	0	no	2	0.07
gi|256083084		Hypothetical protein	*S. mansoni*	3.12	0	0	no	2	-
gi|35833		Hypothetical protein	*Clonorchis*	128	0	0	no	3	0.07

2222		CLF_105212	*sinensis*						
gi|30848 1396		Hypothetical protein CRE_31323	*Caenorhabditis remanei*	155	0	0	no	3	0.55
gi|35322 9814	Smp_1 26540	Hypothetical protein	*S. mansoni*	6.04	0	0	no	3	-
gi|35064 4622	Smp_1 43410	Hypothetical protein	*S. mansoni*	3.01	1	0	no	2	-
gi|35322 9246	Smp_1 61790	Hypothetical protein	*S. mansoni*	3.64	0	0	no	2	-
gi|25608 1928		Ankyrin 23/unc44	*S. mansoni*	94	0	0	no	2	0.02
gi|25607 5638		Family S9 non-peptidase	*S. mansoni*	111/4 .0	0	0	yes	2/2	0.09
	Smp_0 87250	Putative crp1/csrp1/crip1	*S. mansoni*	110	0	0	no	2	0.37
gi|56759 118		SJCHGC02224 protein	*S. japonicum*	114	0	0	no	2	0.35
gi|76155 306		SJCHGC04730 protein	*S. japonicum*	156	1	0	no	2	1.72
gi|24415 108		Trimeric G-protein alpha	*S. mansoni*	71	0	0	no	2	0.18
gi|56756 497		Unknown	*S. japonicum*	6.42	0	0	no	3	-

[a]Scores with decimals belong to ProteinPilot-derived identifications; scores without decimals belong to MASCOT-derived identifications.
[b]TMs, Nº of transmembrane domains.
[C]GPI, glycosyl-phosphatidyl inositol anchor sites.
[d]PN, Mascot, number of significant peptides identified. Protein Pilot number of peptides identified with a confidence > 99%.

Table 2. Non-redundant host proteins identified on the tegumental surface of adult *Schistosoma bovis* worms. The accession number (NCBI) indicates non-redundant identifications by Mascot and ProteinPilot in the NCBI database.

Accession number (NCBI)	Protein name	Species	Score (Mascot/Pilot)[a]	Predicted TMs[b]	GPI[c]	Signal peptide	PN (Mascot/ Pilot)[d]	emPAI
Blood components (n= 8)								
gi|50015	Apoprotein A-I precursor	*Mus musculus*	72	0	0	yes	2	0.23
gi|33859809	Fibrinogen beta chain	*Mus musculus*	1498/36.5	0	0	yes	19/17	6.95
gi|19527078	Fibrinogen gamma chain	*Mus musculus*	864/15.7	0	0	yes	10/7	1.91
gi|33563252	Fibrinogen alpha chain	*Mus musculus*	684/29.3	0	0	yes	9/13	1.18
gi|122441	Hemoglobin subunit alpha	*Mus musculus*	161/4.3	0	0	no	2/2	0.5
gi|31982300	Hemoglobin subunit beta-1	*Mus musculus*	465/13.5	0	0	no	5/6	3.38
gi|17046471	Transferrin	*Mus musculus*	107/8.47	0	0	yes	3/5	0.18
gi|116961	Serine protease inhibitor A3K	*Mus musculus*	120	0	0	yes	2	0.15
Immune response components (n= 11)								
gi|50513865	Anti-Lewis X Fab Fragment Uncomplexed	*Mus musculus*	201	0	0	no	4	0.61
gi|6679457	Bone marrow proteoglycan	*Mus musculus*	115/3.3	0	0	yes	2/3	0.46
gi|83753518	Chain A, Fab Fragment (Antibody 15a9)	*Mus musculus*	201	0	0	no	4	0.7
gi|42543135	Chain A. Model Of Mouse Crry-Ig	*Mus musculus*	309	0	0	no	5	0.75
gi|3114396	Chain L, Domain FAB COMPLEX	*Mus musculus*	214/8.0	0	0	no	5/4	0.93
gi|309266718	Complement C3-like	*Mus musculus*	91	0	0	no	3	0.08
gi|94409900	Complement C4-B-like	*Mus musculus*	333/8.5	0	0	yes	7/4	0.16
gi|90956	Ig mu chain C region	*Mus musculus*	179/6.3	0	0	no	3/3	0.21
gi|194434	Immunoglobulin gamma-chain	*Mus musculus*	355	0	0	no	5	2.37
gi|89477420	mAb5C2 immunoglobulin gamma heavy chain	*Mus musculus*	6.6	0	0	no	3	-
gi|7439167	Monoclonal antibody 13-1 heavy chain	*Mus musculus*	345	0	0	no	5	0.89
Vascular endothelium and leukocyte proteins (n= 6)								
gi|2808470	Basigin	*Mus musculus*	77/3.2	1	0	no	2/2	0.23
gi|53674	CD44	*Mus musculus*	100	1	0	yes	2	0.13

gi|67536 74	Dipeptidyl peptidase 4 isoform 1	*Mus musculus*	97	1	0	no	2	0.08
gi|67543 82	Leukocyte surface antigen CD47	*Mus musculus*	156/4.0	5	0	yes	2/2	0.19
gi|39801 75	Thy 1.2 antigen (CD90)	*Mus musculus*	126/3.8	0	0	no	2/2	0.6
gi|67559 87	Vitronectin precursor	*Mus musculus*	4.0	0	0	yes	2	-
Other (n= 3)								
gi|14866 4711	mCG1031578	*Mus musculus*	277	0	0	no	3	0.89
gi|30239 3781	Ubiquitin	*Cricetulus griseus*	274	0	0	no	5	0.19
gi|74190 108	Unnamed protein product	*Mus musculus*	8.2	1	0	no	3	-

[a]Scores with decimals belong to Protein Pilot-derived identifications; scores without decimals belong to MASCOT-derived identifications.

[b]TMs, Nº of transmembrane domains.

[C]GPI, glycosyl-phosphatidyl anchor inositol sites.

[d]PN, Mascot, number of significant peptides identified. Protein Pilot number of peptides identified with a confidence > 99%.

Table 3. Parasite proteins identified in the tegument of *Schistosoma bovis* adult worms compared to those found in other proteomic studies of the schistosoma tegument surface.

Proteins identified on the tegument surface of *S. bovis*	**Identified in other proteomic studies***	
	Enzymatic shaving	**Biotinylation**
Cytosolic proteins		
14-3-3 protein (épsilon and zeta isoforms)	1a, 2	3
Clathrin heavy chain	-	-
Elongation factor-1 alpha	1a, 2	3
Endophilin B1	1b	-
Fatty acid binding protein 15	1b, 2	-
Fructose bisphosphate aldolase	1a, 1b, 2	-
Glucose regulated protein GRP78	-	-
Glutathione-S transferase 28kda	1a, 1b, 2	3
Glycogen phosphorylase	2	-
Guanosine-diphosphatase	-	-
Heat shock protein 90	1b	3
L-lactate dehydrogenase	1b	-
Major egg antigen (p40)	1b, 2	-
Malate dehydrogenase	-	-
Phosphoglycerate kinase	1b, 2	-
Phosphoglycerate mutase	1b	3
Pyruvate kinase	1b	-
Rab-2414	-	-
Triose phosphate isomerase	1a	-
Cytoskeletal proteins		
Actin	1a, 1b, 2	3,4
Filamin	-	-
Fimbrin	1b	4
Kinesin heavy chain	-	-
Lamin, putative	-	-
Myosin light chain	2	-
Myosin regulatory light chain 2	-	-
Paramyosin	2	-
Spectrin beta chain brain 3	-	-
Tropomyosin	2	-
Troponin T	2	-

Tubulin beta	1b, 2	3
Tubulin alpha-3 chain-like	1b, 2	3
Membrane and tegument-associated proteins		
20 kDa calcium-binding protein (Sm20,0)	2	-
22.6 kDa tegument-associated antigen	1a, 1b	3
ADPATP carrier protein	-	3
Annexin	1b	3, 4
ATPase	-	3
ATP-diphosphohydrolase 1	-	4
Calcium atpase 2	-	3
Calcium-transporting atpase	-	3
Calpain (Smp_80)	1b, 2	3, 4
Cell polarity protein	-	-
Dysferlin1	-	3, 4
Enolase	1a, 1b, 2	3
Glyceraldehyde 3-phosphate dehydrogenase	1b, 2	3, 4
Heat shock protein 70	1a, 1b, 2	3, 4
Innexin	2	-
Phosphodiesterase	-	4
Plasma membrane calcium-transporting atpase	-	3
Rab15 13 10 1 35 5 and	-	-
Snak1		3, 4
Sodium bicarbonate cotransporter	-	-
Surface protein	1a, 1b, 2	3, 4
Tegumental protein Sm 20,8	2	-

***1a** and **1b**, *S. mansoni* adult worms treated with phosphatidylinositol-specific phospholipase C (PiPLC) or trypsin, respectively [19]; **2**, *S. bovis* adult male and female treated with trypsin, (soft protocol, assay 3) [17]; **3**, Biotinylated *S. japonicum* adult worms [18]; **4**, Biotinylated *S. mansoni* adult worms [15].

Significance

To identify the proteins expressed on the surface of the tegument of *Schistosoma bovis* adult worms we used a method based on *in vivo* vascular perfusion, with biotin, of mice infected with *S. bovis* which allowed the labelling of the surface of the worms inside the blood vasculature. This methodology prevents the handling of worms *in vitro* and hence possible damage to the tegument that could produce results that would be difficult to interpret. This work is the first in which vascular perfusion has been used to investigate, in vivo, the protein exposed by an intravascular pathogen on its surface to the host, and provides valuable information about the exposed protein repertoire of the tegument of *S. bovis* in the environmental conditions that the parasite faces inside the blood vessels.

Highlights

- We have analysed the tegument surface of *S. bovis* after labelling of parasites inside blood vessels of infected mice.
- We have identified 80 parasite proteins and 28 host proteins exposed to host on the *S. bovis* tegument.
- This is the first work carried out in which vascular perfusion has been used to investigate an intravascular pathogen.